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Changes in cell wall polysaccharides from ripening olive fruits

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Cell wall material was isolated as alcohol insoluble solids (AIS) from olives at three stages of ripeness. AIS contained 38.8 to 45.6% carbohydrates and 21.5 to 24.8% proteins. Glucose, xylose, arabinose and uronic acids were the major constituent sugars. AIS was sequentially extracted with hot buffer (HBSS), chelating agent (ChSS), dilute alkali (DASS), 1 M alkali (1 M ASS), 4 M alkali (4 M ASS), 4 M alkali + borate (A/BO_3^{3-}), 6 M alkali (6 M ASS) and water (WSS) to leave a cellulose-rich residue (RES). These extracts were characterised by their sugar composition and their molecular weight distribution. The yields, sugar composition, degree of branching and molecular weight distribution of the pectin-rich extracts (HBSS, ChSS, DASS and 6 M ASS) changed during ripening, whereas no significant changes were detected in the other extracts. The degree of branching of the pectins increased with increasing strength of the extractant; the ratio ara:uronic acids increased from 0.46 to 2.59. These extracts were further characterised by their degradability with polygalacturonase (PG) and rhamnogalacturonase (RGase) after chemical saponification. The digests were analysed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC). The amount of oligomers released by PG from pectic material in olive fruits of corresponding extracts from various stages of ripeness decreased with increasing ripening stage. Oligomers released by RGase were formed in similar amounts from corresponding extracts from various stages of ripeness as well as from each extract. HBSS and 6 M ASS were fractionated by anion exchange chromatography. Five pools were obtained for each fractionated extract. HBSS was rich in strongly bound, uronic acid-rich polysaccharides, whereas 6 M ASS was rich in unbound, neutral sugar-rich polysaccharides. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Olive oil is a major product of Mediterranean countries. The mature olive fruit may reach an oil content of 20% on fresh weight basis in about 6–8 months after full bloom. In the course of ripening the colour of the olives changes from green to purple. The olives used for oil production are picked in three time periods and experience has taught that the characteristics of the olives differ between the olives at different stages of ripening.

It was thought that the olive oil yield could be increased by the addition of exogenous enzymatic preparations to the olive paste. Reported results have shown that addition of exogenous enzymatic preparations to the olive paste could increase the oil yield 4–7%, depending on the degree of ripening. The qualitative characteristics of the oil, obtained from olive paste treated with enzymes, were shown to be unchanged or even improved in particular as far as phenolic content and stability to oxidation are concerned (di Giovacchino, 1990). Similar results were obtained by Servili *et al.* (1992); an increase in oil yield was achieved by treatment with a yeast pectinase and the oil quality was generally better.

The enzyme preparations used in these investigations contained mainly polysaccharide degrading enzymes. To determine the optimum composition of an enzyme mixture to be used as an aid in the mechanical extraction of olive oil for the purpose of improving its sensorial, nutritional and keeping qualities, knowledge of the polysaccharide composition of olive cell walls is of great importance.

Some work has already been carried out in order to characterise the individual cell wall polysaccharides extracted from fresh olives. A 4-*O*-methyl glucurono-

xylan was isolated and partially characterised by Gil-Serrano *et al.* (1986). In 1994, Coimbra *et al.* described the isolation of cell wall material from olive pulp, followed by sequential extraction with a number of specific extractants and the isolation and characterisation of glucuronoxylans from the 1 M KOH 1°C extract. We report here on the isolation and characterisation of olive cell wall polysaccharides and changes herein that occur during ripening.

MATERIALS AND METHODS

Plant material

Olive (*Olea europaea* cv Frantoio) fruits of three stages of ripeness, mature green (FI), changing colour (FII) and purple (FIII) were kindly supplied by Professor G.-F. Montedoro and Dr M. Servili, Perugia, Italy.

Isolation of alcohol insoluble solids (AIS)

Olives (1500 g) were boiled in water for 10 min and destoned. The pulp was freeze dried and defatted by the Soxhlet method using petroleum-ether (40–60). The resulting material was extracted with 70% (v/v) ethanol at 40°C for 1 h. The residue was centrifuged and the extraction was repeated until extracts were free of sugars as monitored by the phenol-sulphuric acid test (Dubois *et al.*, 1956). AIS were dried by solvent exchange (96% ethanol and acetone), dried at room temperature and ground (particle size < 1 mm).

Sequential extraction of AIS

Sequential extraction was performed as described by Redgwell and Selvendran (1986) extended to include an extraction with 6 M NaOH (Edelmann & Fry, 1992). AIS (20 g) was sequentially extracted with 0.05 M NaAc buffer, pH 5.2 (three times 600 ml) at 70°C for 30 min (hot buffer soluble solids, HBSS); 0.05 M 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) and 0.05 M NH_4-oxalate in 0.05 M NaAc-buffer, pH 5.2 (two times 600 ml) at 70°C for 30 min (Chelating Agent Soluble Solids, ChSS); washed with distilled water; extracted with 0.05 M NaOH (two times 600 ml) at 4°C for 30 min (dilute alkali soluble solids, DASS); 1.0 M KOH + 20 mM $NaBH_4$ (two times 600 ml) at room temperature for 2 h (1 M alkali soluble solids, 1 M ASS); 4 M KOH +20 mM $NaBH_4$ (600 ml) at room temperature for 2 h (4 M alkali soluble solids, 4 M ASS); 4 M KOH +4% H_3BO_3 +20 mM $NaBH_4$ (600 ml) at room temperature for 2 h (alkali/borate soluble solids, A/BO_3^{3-} SS); 6 M NaOH (600 ml) at room temperature for 2 h (6 M alkali soluble solids, 6 M ASS); destilled water (600 ml) at room temperature for 90 h (water soluble solids, WSS). After each extraction, solubilised polymers were separated from the insoluble residue by centrifugation and the supernatant was filtered through a G3 glass sinter. All extracts were acidified to pH 5.2 (if necessary) by adding glacial acetic acid, concentrated, dialysed and freeze dried. ChSS were dialysed against 0.1 M NH_4Ac buffer, pH 5.2, before dialysing against destilled water. The final residue (RES) was suspended in water, acidified to pH 5.2, dialysed and freeze dried.

Degradation of pectin-rich extracts by PG and RGase

Polygalacturonase (PG; E.C. 3.2.1.15), isolated from *Kluveromyces fragiles* (Schols *et al.*, 1990b), and rhamnogalacturonase (RGase), isolated from *Aspergillus aculeatus* (Schols *et al.*, 1990a, b) were used, to determine the fine structure of the various pectin-rich extracts. The degradation of HBSS and ChSS by these enzymes was performed after saponification, by dissolving them in 0.1 M NaOH (0°C, 16 h) and neutralisation with 0.1 M HAc. Solutions of the samples (0.25%) in 0.05 M NaAc (pH 5.0) were incubated with PG and RGase for 20 h at 30°C, rotating 'head over tail'. The amounts of enzymes added were theoretically sufficient to achieve total degradation of the specific substrate present, in 5 h. After incubation the enzymes were inactivated (10 min, 100°C) and the digests were analysed by HPSEC, HPAEC and sugar composition.

Analytical methods

Protein content was determined by a semi-automated micro-Kjeldahl method (Roozen & van Boxtel, 1979). The conversion factor used was 6.25.

Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples were treated with 72% w/w H_2SO_4 (1 h, 30°C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C and the constituent sugars released were analysed as their alditol acetates. Cellulosic glucose was calculated as the difference between the content of glucose found with and without prehydrolysis.

Uronic acid content was determined as anhydrouronic acid (AUA) content by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979 and Tollier & Robin, 1979) using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample.

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300 × 7.5 mm) of Bio-Gel TSK in series (60XL, 40XL and 30XL; Bio-Rad Labs), in combination with a TSK XL guard column (40 × 6 mm) and elution at 30°C with

0.4 M NaAc buffer pH 3.0 at 0.8 ml/min. Calibration was performed using dextrans. The eluate was monitored using a Shodex SE-61 Refractive Index detector.

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC system as described by Schols *et al.* (1994). The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M NaAc in 0.1 M NaOH.

For the analysis of the PG-digests the (4 × 250 mm) CarboPac PA1 column was equilibrated with 0.2 M NaAc in 0.1 M NaOH. 20 µl of the sample was injected and a linear gradient to 0.6 M NaAc in 0.1 M NaOH was applied. The column was washed for 5 min with 1 M NaAc in 0.1 M NaOH and equilibrated again for 15 min with 0.2 M NaAc in 0.1 M NaOH.

For the analysis of the RGase-digests the column was equilibrated with 0.1 M NaAc in 0.1 M NaOH. 20 µl of the sample was injected and a linear gradient to 0.35 M NaAc in 0.1 M NaOH. The column was washed for 5 minutes with 1 M NaAc in 0.1 M NaOH and equilibrated again for 15 minutes with 0.1 M NaAc in 0.1 M NaOH.

Ion-exchange chromatography: HBSS FI, HBSS FIII, 6 M ASS FI and 6 M ASS FIII were fractionated on a column (275 mm × 23 mm) of DEAE Sepharose fast flow using a Hiload System (Pharmacia), which was initially equilibrated in 0.005 M NaAc-buffer pH 5.0. The polysaccharides in HBSS were saponified as described above. The 6 M ASS fractions were suspended in 50 mM NaAc buffer pH 5.0. The insoluble residues were removed by centrifugation and the supernatants were applied onto the column. Elution was carried out sequentially with 60 ml of 0.005 M NaAc buffer pH 5.0, 400 ml of a linear gradient 0.005–1 M NaAc buffer pH 5.0, 40 ml 1 M NaAc buffer pH 5.0, 40 ml of a linear gradient 1–2 M NaAc buffer pH 5.0, 40 ml 2 M NaAc buffer pH 5.0 and 75 ml 0.005 M NaAc buffer pH 5.0. Residual bound polysaccharides were washed from the column with 80 ml of 0.5 M NaOH and 75 ml 0.005 M NaAc buffer pH 5.0. The elution rate was 5 ml/min except for the first step, in which the rate was 1 ml/min. Fractions (5 ml) were collected and assayed by automated methods (Thibault, 1979 and Tollier & Robin, 1979) for total neutral sugar content and uronic acid content. The appropriate fractions were pooled, concentrated, dialysed, freeze dried and analysed for neutral sugar composition and uronic acid content.

RESULTS AND DISCUSSION

Yield and composition of the AIS

The yields and the compositions of the AISs obtained from olive fruits of the three stages of ripeness are shown in Table 1. The yield of AIS expressed as a percentage of the fresh weight of destoned olive pulp decreased from 8.3% for green olives to 7.4% for purple olives. With increasing degree of ripeness, the ratio pulp to stones increased from 2.5 to 3.1. This can partially be explained by the increase in the oil content of the destoned olive pulp from 27 to 34%. The moisture content of the destoned pulp, however, decreased from 56 to 51%. This means that the relative amounts of components other than oil also increased during ripening.

The yields of AISs found here were higher than those reported by Coimbra *et al.* (1994) for CWM (cell wall material) isolated from olives of the Douro variety (3.7% on fresh weight basis). The carbohydrate content of AIS decreased from 45.6% for AIS FI to 38.8% for AIS FIII, which was less than that of CWM (61.9%) isolated by Coimbra *et al.* (1994). This difference can partly be ascribed to the different varieties of olives used and to the fact that Coimbra *et al.* (1994) used another method to isolate cell wall material. AIS still contained water soluble material and CWM did not, and extraction with SDS and PAW (phenol–acetic acid–water) treatment by Coimbra *et al.* removed the bulk of the protein, which resulted in a protein content of 8.6% in CWM. Thus in our method of extracting AIS, more other components — like water soluble carbohydrates and protein — are included. The protein and the carbohydrate content of AIS accounted for 63–70% of AIS, the ash content was approximately 1% and the moisture content was 3.5–4.5%, the remaining 25–32% consisted partly of lignin-like polyphenolic material.

The carbohydrate composition of AIS is also shown in Table 1. Arabinose, xylose and anhydro-uronic acids each accounted for about 20%, and glucose for about 35% of the carbohydrates of olive AIS, while rhamnose, mannose and galactose were present in relatively small

Table 1. Yield and composition of olive AIS at the three stages of ripeness

Sample	Yield[a]	Sugar composition[b]							Carbohydrate content[c]	Protein content[c]
		rha	ara	xyl	man	gal	glc	AUA		
FI	8.3	2	20	23	2	3	34	17	45.6	21.5
FII	7.4	2	18	20	2	3	36	19	44.4	24.8
FIII	7.4	2	16	21	2	3	39	18	38.8	24.2

[a]xpressed as % of fresh weight of destoned olive pulp; [b]expressed as mol%; [c]expressed as % w/w.

amounts. The sugar composition of CWM isolated by Coimbra *et al.* (1994) can be compared to that of AIS FI, because CWM was isolated from olive fruits harvested at the green mature stage as were the olives of stage FI. The results agree rather well, CWM was also low in rhamnose, mannose and galactose, but it contained more arabinose and anhydro-uronic acids and less xylose and glucose. These differences may be due to the different varieties of olives used.

During ripening the arabinose content decreased from 20% to 16%, the xylose content from 23 to 21% and the glucose content increased from 34% to 39%. The increase in glucose content is partially due to the increase in cellulose content. Although the differences are relatively small, they are good reproducible and consistent. The relative amounts of rhamnose, mannose, galactose and AUA did not change during ripening of these olives.

The changes that occur during ripening of the olives do not find expression in the composition of AIS. Possible changes in the individual polysaccharides would not always be detected in the overall sugar composition. Therefore, AIS from the two extreme stages of the ripening spectrum (FI and FIII) are sequentially extracted with specific aqueous extractants to isolate the different groups of polysaccharides.

Characterisation of the extracts of AIS FI and AIS FIII

The yields and the sugar compositions of the extracts of AIS FI and AIS FIII are presented in Tables 2 and 3, respectively. The sugar composition expressed as both mol% and mg/g AIS are shown. The first two successive treatments extracted much of the uronic acids present in AIS FI and AIS FIII. Although AUA is a major constituent of the DASS fractions, the concentrations in DASS are only 6.8 and 6.1 mg/g AIS for FI and FIII, respectively, so it represents only about 7.5% of the uronic acids present in AIS. Besides these uronic acids, these two extracts had a high arabinose content in addition to small amounts of rhamnose, indicating the presence of branched pectins in these extracts. It can be seen that the ratio neutral sugars:uronic acids increased when more severe extraction conditions were used.

The fractions extracted from the residue that remained after extraction with 4 M alkali + borate (6 M ASS and WSS) were rich in arabinose, uronic acids and contained some rhamnose. It seems that during these two extractions also pectic materials were released. This is in accordance with the results of Coimbra *et al.* (1994); they found that the supernatant solution, obtained after neutralisation and dialyses of the residue remaining after extraction with 4 M KOH + borate, was rich in pectic polysaccharides. The extraction of cell wall material with 6 M alkali is related to the swelling of cellulose (Edelmann & Fry, 1992), but alkali can also effect cell wall-bound pectins in a way that renders them subsequently extractable in water (Shedletzky *et al.*, 1990). The WSS fractions contained 53% (WSS FI) and 33% (WSS FIII) of xylose, part of this might be present as glucuronoxylan as described by Coimbra *et al.* (1994), while also some (glucurono)arabinoxylan might be present in these extracts.

Pectin

Pectic polysaccharides are considered to contain homogalacturonan regions — smooth regions — and so-called hairy regions. These hairy regions contain neutral sugar side chains of (arabino)galactans and arabinans (de Vries *et al.*, 1982). These hairy regions have been reported to be present in many plant tissues. Glycosidic-linkage analysis of a major CDTA sub-fraction by Coimbra *et al.* (1994) showed the presence of small amounts of terminal galacturonic acid residues in addition to (1,4)-linked Gal*p*A, variously linked rhamnose and arabinose residues and a small amount of variously linked galactose residues. This corresponds to the more general model of pectins, except for a low content of galactose in the side chains. Therefore, the ratio ara:AUA is used as an indication for the degree of branching of the pectins in this study.

In the pectin-rich extracts — HBSS, ChSS, DASS and 6 M ASS — the changes in arabinose and uronic acid content during ripening are obvious. In the HBSS fractions the rhamnose content increased from 2 to 3 mol%, the arabinose content increased from 26 to 32 mol% and the uronic acid content decreased from 57 to 50 mol%. The ratio ara:AUA changed from 0.46 for HBSS FI to 0.64 for HBSS FIII, indicating that the pectins extracted with hot buffer from AIS FIII are more branched or contained longer side chains than those extracted from AIS FI. In ChSS, only slight changes in the sugar composition could be determined. The ratio ara:AUA increased from 0.63 for ChSS FI to 0.67 for ChSS FIII, it seems that the degree of branching of the pectins extracted with chelating reagent from AIS FIII is similar to that of the pectins extracted from AIS FI. Only small differences were found in the sugar compositions of DASS FI and DASS FIII. The ratio ara:AUA changed from 1.43 for DASS FI to 1.55 for DASS FIII, this indicates that the pectins extracted with DASS from AIS FIII are slightly more branched or contain slightly longer side chains than those extracted from AIS FI. In the final pectin-rich extract, 6 M ASS, the ratio ara:AUA showed a slight increase from 2.59 for 6 M ASS FI to 2.63 for AIS FIII. This indicates that the degree of branching is quite similar for these two extracts.

Changes occurring during ripening, resulted in changes in the molecular weight distributions of the pectin-rich extracts HBSS, ChSS, 6 M ASS and in a lesser extend in DASS (Fig. 1). The elution patterns of HBSS

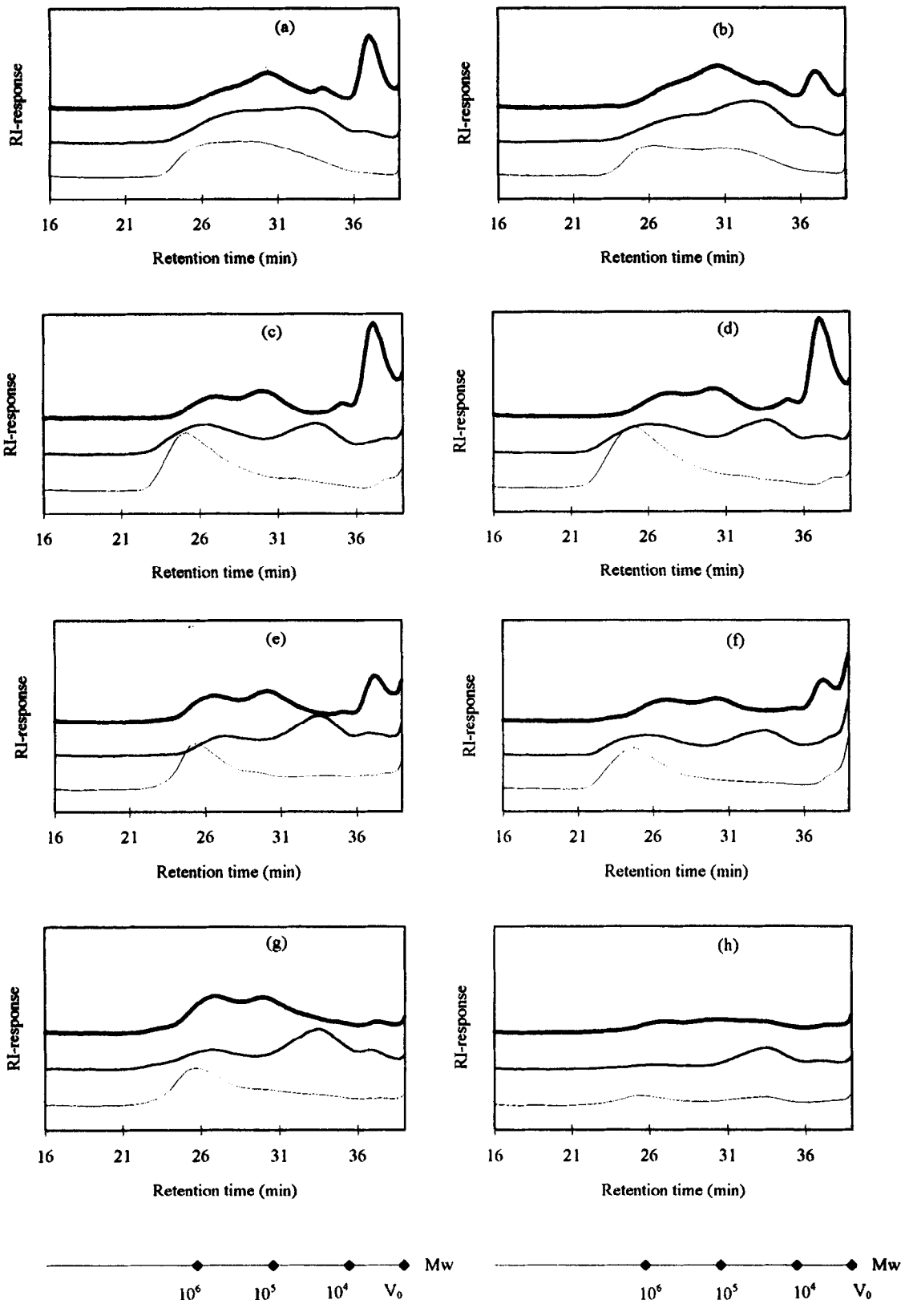

Fig. 1. Degradation of saponified: (a) HBSS FI; (b) HBSS FIII; (c) ChSS FI; (d) ChSS FIII; (e) DASS FI; (f) DASS FIII; (g) 6M ASS FI and (h) 6 M ASS FIII by PG (——) abd RGase (——), followed with HPSEC [blank (——)]. Calibration was performed using dextrans.

show a broad molecular weight distribution. During ripening a shift to lower molecular weight material is noticeable, two peaks with different molecular weights appear. ChSS elution patterns consist of a broad peak in the high molecular weight area. During ripening, the shape of the peak does not change, but the peak maximum slightly increases and some higher molecular weight material was extracted, as can be seen by the slightly earlier peak start. The DASS fractions show a peak in the same molecular weight area as ChSS. At the third stage of ripening, the molecular weight distribution became broader, due to the extraction of very high molecular weight material. The elution pattern of 6 M ASS FI shows an obvious peak at high molecular weight whereas 6 M ASS FIII is hardly soluble, two small broad peaks are noticeable. The HPSEC elution patterns of WSS (not shown) show a broad molecular weight distribution at all stages of ripening. The pattern did not change, but peak height and area decreased during ripening. This is probable due to the lower total sugar content of WSS FIII (13.6%) compared to that of WSS FI (47.7%), since we standardised on equal amounts of extract per ml.

Hemicellulose

In the hemicellulose-rich extracts — 1 M ASS, 4 M ASS and A/BO_3^{3-} SS — the prevailing sugars were xylose, glucose, arabinose and uronic acids. The uronide material present in these extracts is presumably glucuronic acid, originating from a glucuronoxylan (Coimbra *et al.*, 1994).

The 1 M ASS fractions contained about 50 mol% of xylose and considerable amounts of arabinose, AUA and glucose (10–18%). The major part of the polysaccharides in this extract is probably present as glucuronoarabinoxylans. During ripening the xylose content showed a small decrease from 55 to 49 mol%, the arabinose content increased from 9 to 16 mol% and the AUA content decreased from 18 to 12 mol%. The polysaccharides in this extract probably do not change very much during ripening, because arabinose and AUA are only minor constituents.

The 4 M ASS fractions mainly consisted of xylose and glucose, and these two sugars accounted for about 65% of the total sugars present. So it is likely that a xyloglucan, which is common to the primary walls of higher plants (Hayashi, 1989), is present in this extract. 4 M ASS did not show any obvious changes in sugar composition as a result of ripening.

Due to the low total sugar content of the A/BO_3^{3-} fractions (5.3% for FI and 5.9% for FIII) small changes in the sugar composition expressed as % w/w (not shown) seem significant changes when expressed as mol%. For example, the increase in rhamnose from 2 to 7 mol% equals an increase from 0.1 to 0.4% w/w (not shown). Therefore the data obtained for these extracts should be interpreted very carefully.

The HPSEC elution patterns of 1 M ASS, 4 M ASS (not shown) both showed two populations of polysaccharides present and only minor differences during ripening were seen. The A/BO_3^{3-} fraction did not dissolve, so peak(s) were hardly noticeable.

Cellulose

The final residue after extractions with 6 M NaOH and distilled water gave yields of 54 and 48% of AIS on weight basis, and contained 63 and 65 mol% glucose. The residues contained moderate amounts of 1 M H_2SO_4-hydrolysable glucose, namely 10%. This glucose was probably derived from a hemicellulosic glucan that is either covalently linked to, or so extensively hydrogen-bonded to the cellulose that it resisted release by 6 M NaOH.

When the data in Tables 2 and 3 are compared, it is noticed that the sugar composition of the final residue (RES) did not change during ripening. Besides 63–65% glucose it contained 20–23% xylose and 7–10% uronic acids, other sugars were present in minor quantities.

Changes during ripening

During ripening the olive fruits change colour, the oil content increases and the fruit looses firmness. The latter is partially due to modification of the cell wall components, caused by endogenous enzymes and deposition of new polymers. Heredia *et al.* (1990, 1993) have shown a considerable increase in cellulase activity of olive fruits during ripening and softening and the development of several glycosidases.

As appears from the results reported above, the changes in the cell wall composition of olives during ripening take place mainly in pectins. The yields, sugar compositions, branching and molecular weight distributions of the pectin-rich extracts undergo changes as the olives ripen. Biale and Young (1981) already mentioned the major role of pectic substances in softening of fruit tissue.

The pectins in 6 M ASS are highly branched and very difficult to extract from the cell wall material. A 6 M NaOH solution was needed for the extraction of these pectins, which means that they are either tightly bound to the cellulose/xyloglucan network or they are enclosed in this network. The yield of 6 M ASS decreased from 7.4% for 6 M ASS FI to 5.3% for 6 M ASS FIII. A possible explanation is the enzymatic degradation of cell wall material during ripening. These enzymes can either hydrolyse the bonds between these highly branched pectins and the cellulose/xyloglucan network or loosen up (parts of) this network. This can lead to the improved extractability of these branched pectins, and thus to a decrease of the yield of 6 M ASS.

The yield of pectins extracted with hot buffer increased during ripening from 9.4 to 10.3% and the degree of branching also increased. Possibly (part of) the pectins, which were difficult to extract from AIS

Table 2. Yield on sugar basis (%) and sugar composition of extracts of AIS FI expressed as mol%, and as mg/g AIS in parentheses

Extract	Yield	rha	ara	xyl	man	gal	glc	AUA	Carbohydrate content[a]
AIS FI	100	2 (7)	20 (81)	23 (89)	2 (8)	3 (15)	34 (165)	17 (92)	45.6
HBSS FI	9.4	2 (1.2)	26 (14.7)	2 (1.3)	1 (0.7)	8 (5.5)	4 (2.7)	57 (42.9)	73.4
ChSS FI	3.9	3 (0.6)	34 (7.3)	1 (0.2)	1 (0.2)	4 (1.0)	5 (1.2)	54 (15.6)	67.0
DASS FI	3.6	3 (0.4)	50 (7.2)	2 (0.4)	t (0.1)	7 (1.2)	3 (0.5)	35 (6.8)	45.8
1 M ASS FI	8.8	1 (0.3)	9 (3.4)	55 (20.7)	1 (0.5)	5 (2.5)	10 (4.7)	18 (9.1)	46.6
4 M ASS FI	5.3	t (0.1)	7 (1.4)	40 (7.7)	8 (1.8)	7 (1.6)	24 (5.6)	13 (3.4)	41.0
A/BO_3^{3-} SS FI	1.9	2 (t)	57 (0.5)	10 (0.1)	2 (t)	5 (0.1)	10 (0.1)	15 (0.2)	5.3
6 M ASS FI	7.4	3 (1.4)	57 (24.3)	4 (1.6)	2 (1.2)	6 (2.9)	6 (3.1)	22 (12.7)	63.6
WSS FI	1.4	1 (0.1)	27 (1.7)	53 (3.3)	0 (0)	1 (0.1)	3 (0.2)	15 (1.3)	47.7
RES FI	53.5	t (1.6)	4 (12.8)	23 (78.1)	0 (0)	t (t)	63 (258.9)	10 (42.8)	73.5

t = Trace amount; [a]expressed as % w/w.

Table 3. Yield on sugar basis (%) and sugar composition of extracts of AIS FIII expressed as mol%, and as mg/g AIS in parentheses

Extract	Yield	rha	ara	xyl	man	gal	glc	AUA	Carbohydrate content[a]
AIS FIII	100	2 (6)	16 (53)	21 (69)	2 (7)	3 (13)	39 (157)	18 (82)	38.8
HBSS FIII	10.3	3 (2.0)	32 (20.6)	3 (1.9)	1 (0.7)	8 (5.9)	4 (3.5)	50 (43.1)	75.4
ChSS FIII	7.0	4 (1.6)	37 (15.6)	1 (0.2)	t (0.2)	3 (1.7)	1 (0.4)	55 (30.7)	71.9
DASS FIII	3.4	4 (0.6)	51 (7.0)	3 (0.3)	0 (0)	6 (1.0)	3 (0.5)	33 (6.1)	45.8
1 M ASS FIII	9.2	2 (0.6)	16 (4.6)	49 (14.6)	2 (0.7)	7 (2.4)	13 (4.7)	12 (4.8)	35.3
4 M ASS FIII	7.3	1 (0.2)	11 (1.9)	39 (7.0)	7 (1.6)	6 (1.4)	26 (5.7)	9 (2.3)	27.5
A/BO_3^{3-} SS FIII	1.8	7 (0.1)	42 (0.4)	9 (0.1)	4 (0.1)	5 (0.1)	15 (0.2)	18 (0.2)	5.9
6 M ASS FIII	5.3	3 (0.4)	50 (6.6)	3 (0.5)	8 (1.3)	6 (1.0)	11 (1.7)	19 (3.4)	28.2
WSS FIII	1.3	2 (t)	43 (0.7)	33 (0.5)	1 (t)	2 (t)	5 (0.1)	14 (0.3)	13.6
RES FIII	48.1	1 (1.9)	6 (15.9)	20 (55.3)	t (1.4)	1 (2.4)	65 (217.8)	7 (25.5)	66.6

t = Trace amount; [a]expressed as % w/w.

FIII, are now extracted with hot buffer. This would explain both the increase in the yield and the increase in the ratio ara:AUA.

The yield of ChSS increases from 3.9 to 7.0% during ripening. It is reasonable to suggest that the extra amount of pectin extracted from AIS FIII is newly synthesised, since pectins which become easier extractable during ripening are probably extracted with the hot buffer solution: it is not likely that they will form a complex with Ca^{2+} and need a chelating agent to be extracted. This is confirmed by the fact that the sugar composition and the ratio ara:AUA do not change during ripening. Although it can not be ruled out that PE (pectin-methylesterase) is active during ripening.

The yield of DASS hardly changes during ripening; 3.6% for DASS FI and 3.4% for DASS FIII. The sugar composition and the degree of branching showed little changes, DASS FIII has a slightly higher ara:AUA ratio than DASS FI. These small changes could possibly be explained by enzymatic degradation, which would solubilise a small amount of less-branched material.

The most important changes that occur in cell wall polysaccharides during ripening concern the pectin-rich extracts, so these have to be further characterised. A possible way to make differences visible (even when the sugar composition is similar) is enzymatic degradation of the extracts (Voragen *et al.*, 1993).

Enzymatic degradation of pectin-rich extracts

Further characterisation of the pectin-rich extracts, HBSS, ChSS, DASS and 6 M ASS, was performed by degrading them with PG and RGase. The digests are analysed for their molecular weight distribution (Fig. 1), for the presence of PG and RGase oligomers (Fig. 2) and the sugar composition of the soluble fraction (not shown). These results will lead to partial elucidation of the fine structure of pectins in these extracts.

PG catalyses the hydrolysis of the *O*-glycosidic linkage of α-D-(1,4)-polygalacturonan (Burns, 1991). HBSS and ChSS have to be saponified before incubation, since hydrolysis preferably takes place next to a free carboxyl group.

RGase cleaves galactopyranosyluronic-rhamnopyranosyl linkages within the hairy (ramified) regions of pectin. HBSS and ChSS have to be saponified before incubation, because RGase is hindered by *O*-acetyl groups (Schols *et al.*, 1990a). Characteristic RGase oligomers obtained after degradation of apple MHR (modified hairy regions) by RGase, which consisted of between four and

nine sugar units with a backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose residues linked to C-4 of the rhamnose moiety, are described by Schols *et al.* (1994).

HBSS

Incubation of HBSS FI and HBSS FIII with PG resulted in considerable degradation of these extracts, as is shown in Fig. 1(a) and (b). A peak representing the characteristic PG oligomers, with a retention time of about 37 min, appeared. The formation of these galacturonic acid oligomers was confirmed by HPAEC (Fig. 2A). The amount of PG oligomers in the HBSS FIII digest was lower than in the HBSS FI digest. From the sugar composition it was concluded that only a neutral sugar-rich fraction of the saponified HBSS was soluble, probably due to the formation of a Ca^{2+}-pectate gel. PG was able to increase the solubility of these saponified smooth regions in HBSS FI from 30 to 80% and in HBSS FIII from 40 to 60%. After incubation with PG, the fragments resistant to further degradation remained.

After incubation of HBSS with RGase low molecular weight polymers appearing at 32–33 minutes and some RGase oligomers appearing at about 37 minutes can be detected. The oligomers formed were identified by comparing them with those described by Schols *et al.* (1994). As shown in Fig. 2, oligomers I (Rha_2GalA_2), V (Rha_3GalA_3) and VIII ($Rha_3GalA_3Gal_2$) are present and oligomers II, III (both Rha_2GalA_2Gal with galactose substitution on different rhamnose residues), IV ($Rha_2GalA_2Gal_2$) and IX ($Rha_3GalA_3Gal_3$) can be detected as minor components. The digests from both stages of ripening contained the same RGase oligomers in similar amounts. However, more low molecular weight polymers (retention time 32–33 min) were formed during incubation of HBSS FIII. The sugar composition of the digest is the same as that of the blank, indicating that RGase was not able to solubilise the smooth regions. This means that the soluble hairy regions were degraded, and the insoluble residue and the high molecular weight fraction in the HPSEC chromatograms (remaining after incubation) consist of smooth regions.

ChSS

The degradation of ChSS FI and FIII with RGase and PG resulted in similar digests, as shown in Fig. 1(c) and 1(d), the degree of ripening did not effect the degradation pattern. The peak representing the PG oligomers was confirmed by the presence of these galacturonic acid oligomers as illustrated in Fig. 2. Oligomers consisting of one to four residues are detected, in higher amounts than in the PG digest of HBSS. PG degraded the insoluble saponified smooth regions and increased their solubility from 50 to 100%. The hairy regions remained as a high molecular weight peak in the HPSEC chromatogram.

After incubation of ChSS FI with RGase, the polysaccharides were partly degraded and low molecular

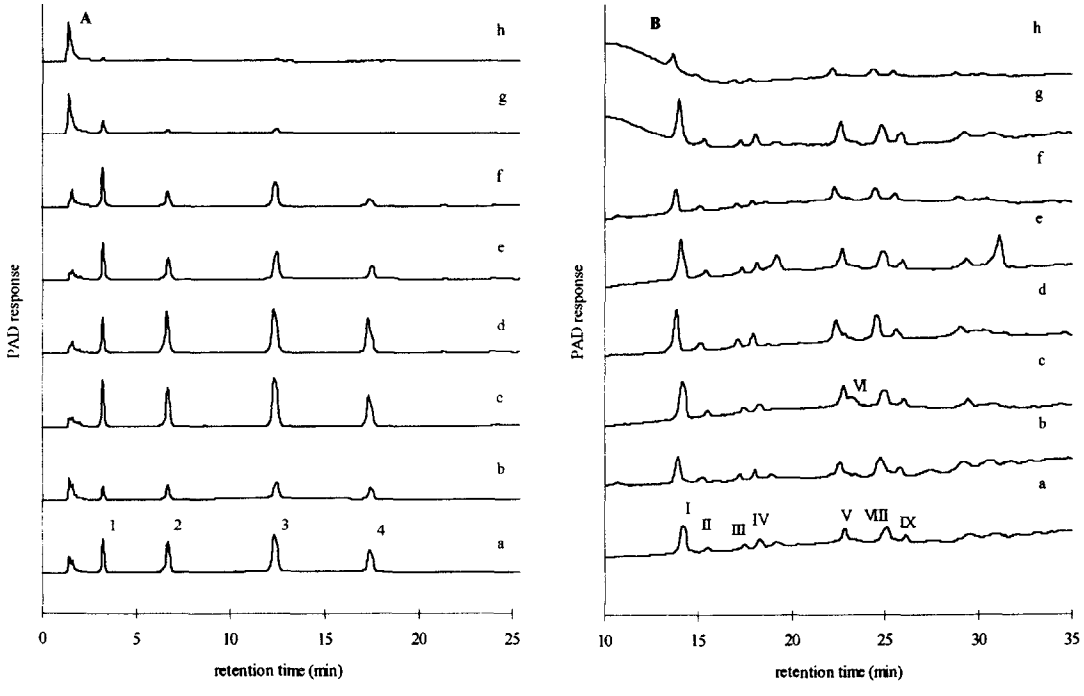

Fig. 2. HPAEC elution patterns of various saponified extracts from olive AIS after treatment with PG (A) and RGase (B). (a) HBSS FI, (b) HBSS FIII, (c) ChSS FI, (d) ChSS FIII, (e) DASS FI, (f) DASS FIII, (g) 6 M ASS FI and (h) 6 M ASS FIII. 1, $GalA$; 2, $GalA_2$; 3, $GalA_3$ and 4, $GalA_4$. I, Rha_2GalA_2; II and III, Rha_2GalA_2Gal; IV, $Rha_2GalA_2Gal_2$; V, Rha_3GalA_3; VI, Rha_3GalA_3Gal; VIII, $Rha_3GalA_3Gal_2$ and IX, $Rha_3GalA_3Gal_3$.

weight polymers and a small amount of RGase oligomers were liberated. In addition to the oligomers released from HBSS FI, oligomer VI (Rha_3GalA_3Gal) can also be detected. RGase solubilised 50–60% of the uronic acids present in the original material. So after incubation some insoluble (residue) and some soluble smooth regions remained.

DASS

The PG digests of DASS FI and FIII showed similar HPSEC elution patterns, with a slightly lower peak for the characteristic PG oligomers in the DASS FIII digest. This is confirmed by the HPAEC pattern in which the four peaks were slightly smaller compared to the pattern of the DASS FI digest.

The treatment of DASS FI with RGase is more effective than the treatments of both HBSS and ChSS. Only little polymeric material remained after incubation and low molecular weight polymers and a small amount of RGase oligomers was formed. The HPAEC elution pattern showed that oligomers I–IX were present in the RGase digest. The oligomers without galactose substitution, I and V, were the most abundant again. RGase is also active on the DASS FIII fraction, but in a lesser extend than on the DASS FI fraction. The low molecular weight polymers at 32–33 min were formed in smaller amounts as is shown by HPSEC analysis and the HPAEC elution patterns show that peaks for the RGase oligomers are also a little smaller.

6M ASS

PG degraded the polysaccharides present in 6 M ASS FI in a very limited extend and hardly degraded 6 M ASS FIII. The rather large fragments formed from 6 M ASS FI elute at 30 minutes, and these fragments were resistant to further degradation by PG. A very small amount of PG oligomers was formed, as shown by both HPSEC and HPAEC. The insoluble polysaccharides of this fraction are partly solubilised by the action of PG.

The RGase digests of 6 M ASS FI and FIII showed a decrease of polymeric material and the presence of typical RGase oligomers. Oligomers I–IX could be detected by HPAEC. The amounts of RGase oligomers in the 6 M ASS FIII digest were smaller than in the RGase digest of 6 M ASS FI, but the same oligomers were still present. Besides, RGase is able to solubilise the insoluble polysaccharides present in the blank by partly degrading these structures.

Changes during ripening

PG was more active on both HBSS FI and FIII than RGase, indicating that smooth regions were more abundant than hairy regions, as was already concluded from the sugar composition. When incubating HBSS FI with PG, more uronic acid oligomers are formed than from HBSS FIII, so the amount of smooth regions decreased during ripening. RGase was more active on HBSS FIII: the amount of polymers with a retention time of about 32–33 min in this digest was higher than in the digest of HBSS FI and oligomers were formed in equal amounts. This is in full agreement with the increase in the ara:AUA ratio of the pectins extracted with hot buffer during ripening, as stated before on account of the results of characterisation of the extracts of olive AIS FI and FIII by HPSEC and their sugar composition.

The incubation of ChSS FIII with RGase and PG resulted in HPSEC elution patterns, HPAEC elution patterns and sugar compositions (not shown) which are similar to that of ChSS FI. This means that during ripening the polysaccharides extracted with ChSS do not change. This is in agreement with the results mentioned before which showed that the sugar composition and the degree of branching do not change.

The polysaccharides extracted with diluted alkali were completely soluble, whereas the smooth regions present in HBSS and ChSS were partly precipitated by saponification. Comparison with HBSS and ChSS shows that RGase is more active and PG is less active on this extract. These two facts suggest that the DASS fractions are probably more branched than HBSS and ChSS, which was already mentioned before. This implication is supported by the increasing ratios of ara:AUA, which are 0.46, 0.63 and 1.43 for HBSS FI, ChSS FI and DASS FI, respectively.

During ripening the solubility of the polysaccharides extracted with 6 M NaOH decreased. From the fact that PG has almost no activity and RGase has, it can be concluded that these polysaccharides must be very branched and even more branched than DASS. This is confirmed by the ratio of ara:AUA of 2.6 as calculated before. It was also stated before that the branching of this extract did not change during ripening, which can neither be confirmed nor defeated by these results.

Fractionation of the pectin-rich extracts, HBSS and 6 M ASS

The polymers present in HBSS and 6 M ASS were subjected to anion-exchange chromatography (Fig. 3) and most of the polysaccharide material was recovered (75–92%). The first pool, which consisted of unbound neutral polysaccharides, contained mainly glucose and was present in all samples. This first pool is only of minor importance in the profiles of HBSS. The bound fractions were eluted from the column with increasing concentration of sodium acetate. The HBSS gave three bound pools and 6 M ASS gave four bound pools. In all cases some material was strongly retained on the column and could not be removed by 2 M sodium acetate buffer. Sodium hydroxide was needed to elute this material (pool V). For every pool

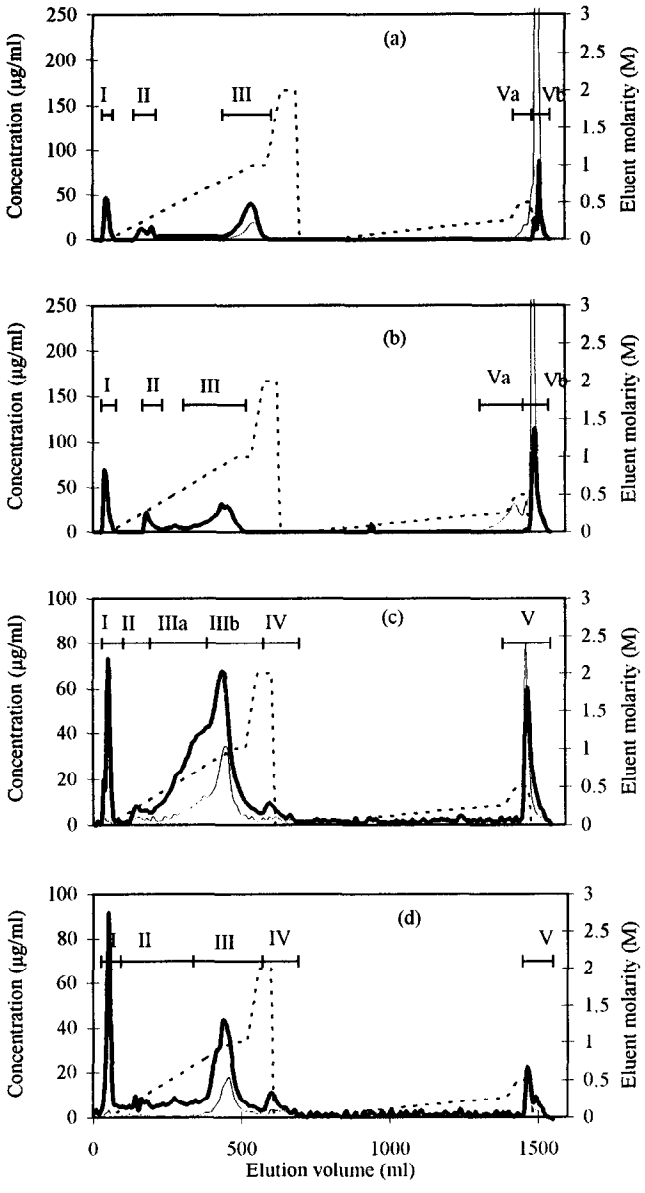

Fig. 3. Elution profiles of saponified (a) HBSS FI; (b) saponified HBSS FIII; (c) 6M ASS FI; and (d) 6 M ASS FIII on DEAE-Sepharose fast flow. Eluent molarity (- - - -), galacturonic acid (——) and neutral sugurs (——).

the sugar composition was determined and shown in Table 4.

Pool I from HBSS contained mainly glucose, galactose, and xylose and pool I from 6 M ASS contained a large amount of mannose in stead of xylose. In the elution pattern of HBSS pool I was of minor importance and contained only minor amounts of arabinose. In the elution pattern of 6 M ASS pool I was a major fraction but also contained only minor amounts of arabinose: 16 and 4% for FI and FIII, respectively. Since all unbound fractions were very poor in uronic acids and no rhamnose was detected, it was shown that almost all arabinose was linked to the pectin which was bound to the anion-exchanger.

Pool II is a minor fraction in all extracts. The sugar composition showed the presence of mainly neutral polysaccharides rich in arabinose, galactose and glucose in HBSS FI and FIII and arabinose, glucose and xylose are the major constituents of 6 M ASS FI and FIII.

Pool III is a minor fraction in HBSS FI and FIII, but a major one in 6 M ASS FI and FIII. These pools contained substantial amounts of uronic acids (17–33%), even more arabinose (51–57%) and some rhamnose (2–3%), all other sugars are present in small amounts. This means that pool III is a pectin-rich pool. The arabinose:uronic acids ratio is approximately 2:1, which suggests the presence of hairy regions.

Pool IV is detected in the elution patterns of 6 M ASS only, where it is a fraction of minor importance. The most abundant sugars are arabinose, xylose and glucose, all other sugars are present as minor components.

Pool V is present in all extracts again. In the elution patterns of HBSS FI and FIII a little shoulder preceding the peak is noticeable and pooled as Va. This fraction contained mainly uronic acids, 74 and 83% in HBSS FI and FIII, respectively. This indicates that these are almost non-branched polygalacturonic acid chains. Pools Vb and V differed from Va, they contained an important amount of arabinose besides uronic acids. The arabinose:uronic acid ratio is 1:3 in HBSS FI, 1:2 in HBSS FIII, 1:1.5 in 6 M ASS FI and 1:1 in 6 M ASS FIII. The relative amount of this fraction in the extracts decreased in this order.

Changes during ripening

These results show that the amount of pool I relatively increased and the amount of pool III decreased in 6 M ASS. Besides that, the polysaccharides in pool V extracted with 6 M alkali are more branched and/or have longer arabinose side chains than those in pool V extracted with hot buffer. During ripening this degree of branching and/or the length of the side chains decreased in both HBSS and 6 M ASS. The ratios found here could indicate a shift from the lesser branched part of the pectins in pool V from 6 M ASS FI to HBSS FIII, which would then lead to both an increase in the ratio arabinose:uronic acids in HBSS and in 6 M ASS. Further research is needed to confirm this supposition.

CONCLUSION

Knowledge of changes in cell wall material from olives during ripening is important in the olive oil industry, for adjusting processing conditions and for determining the optimum composition of the enzyme mixture to add during the mechanical extraction. In this paper, changes during ripening in the pectic

Table 4. Sugar composition expressed as mol% of DEAE fractions of HBSS and 6 M ASS

Extract	Fraction	rha	ara	xyl	man	gal	glc	AUA
HBSS FI	I	0	10	17	6	21	43	2
	II	2	25	10	5	28	25	4
	III	2	51	2	3	5	4	33
	Va	0	5	9	4	1	8	74
	Vb	2	20	2	2	3	5	67
HBSS FIII	I	0	16	19	3	23	37	2
	II	0	32	9	2	36	16	5
	III	3	56	2	1	5	5	27
	Va	1	2	1	3	1	9	83
	Vb	3	31	1	1	4	1	59
6 M ASS FI	I	0	16	4	27	13	36	5
	II	1	44	24	5	3	14	9
	IIIa	2	57	3	1	5	4	28
	IIIb	2	67	4	1	5	5	17
	IV	1	41	10	7	8	25	6
	V	2	36	4	1	5	6	48
6 M ASS FIII	I	0	4	2	33	14	44	4
	II	0	44	11	10	4	22	8
	III	2	52	9	1	5	10	21
	IV	1	40	33	2	5	13	6
	V	2	37	9	2	4	9	37

material isolated from olives are determined. It is shown that pectic material with a high degree of branching is difficult to extract from the cell wall material of green olives. During ripening these pectins become easier extractable.

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